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14. ABSTRACT
Mutations in the BRCA2 gene are linked to familial and sporadic breast cancer, yet the molecular function of BRCA2 protein remains largely obscure. BRCA2 protein physically interacts with the Rad51 recombinase, a member of the RAD52 epistasis group of proteins that mediate homologous recombination (HR), a major mechanism that repairs chromosomes damaged by ionizing radiation and genotoxic agents. Accordingly, BRCA2 deficient cell lines exhibit impaired HR and sensitivity to genotoxic agents. To help define the molecular function of human BRCA2, we have expressed and purified a polypeptide that harbors the BRC3 and BRC4 repeat and also the DNA binding domain of this tumor suppressor. The BRC3/4-DBD polypeptide interacts with hRad51 and binds DNA with a distinct preference for ssDNA. Importantly, we have demonstrated by biochemical means and electron microscopy that BRC3/4-DBD nucleates hRad51 onto ssDNA and acts as a recombination mediator in enabling Rad51 to utilize replication protein A-coated ssDNA as recombination substrate. In isolation neither the BRC3-BRC4 repeats nor the DNA binding domain of BRCA2 performs these mediator functions. The biochemical system described in this study should be valuable for systematically dissecting the HR functions of BRCA2 and its associated proteins such as DSS1. Comprehending the manner in which BRCA2 modulates Rad51 activity and the functional integrity of the homologous recombination machinery could very well pave the way for devising new strategies in breast cancer diagnosis, prevention, and treatment.

15. SUBJECT TERMS
BRCA2, DNA repair, Homologous Recombination, Rad51

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Introduction

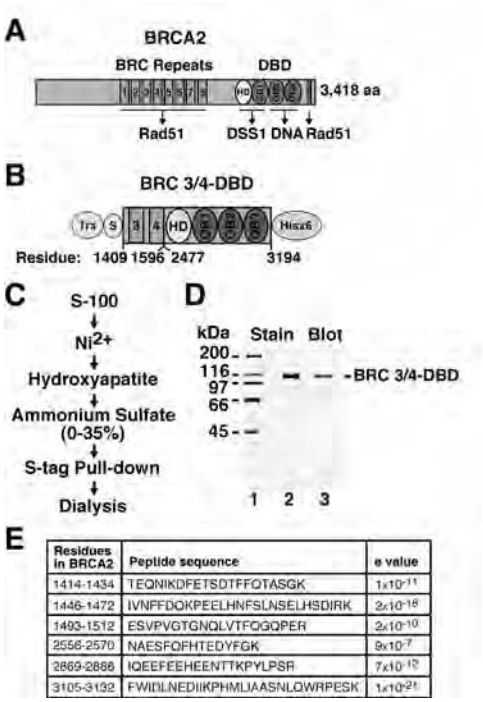
The repair of DNA double-strand breaks (DSB) induced by exogenous agents (e.g. ionizing radiation) or that arise endogenously (e.g. replication of damaged DNA template) is critical for the maintenance of genome stability. In eukaryotes, homologous recombination (HR) represents an important mechanism for the repair of DSBs and is mediated by genes of the RAD52 epistasis group, whose structure and function are highly conserved (Symington, 2002; Sung et al., 2003). In HR-mediated DSB repair, the ends of the DNA break are processed nucleolytically to yield a pair of ssDNA tails, which serve as the nucleation site for the Rad51 recombinase. Polymerization of Rad51 onto the ssDNA results in the formation of a right-handed nucleoprotein filament, also commonly referred to as the presynaptic filament (Symington, 2002; Sung et al., 2003). After locating a DNA homologue, the presynaptic filament catalyzes pairing with the homologue to form a nascent DNA joint called a displacement loop (D-loop), the length of which is extended by DNA strand exchange. Subsequent steps include resolution of recombination intermediates and ligation to complete the recombination/repair reaction.

Mutations in the BRCA2 (breast cancer susceptibility 2) gene are found in a significant portion of familial breast cancer cases and confer an increased risk of ovarian, pancreatic, and prostate cancer (Ford et al., 1998; Wooster et al., 1995). Mutations in BRCA2 can also lead to the cancer prone-syndrome Fanconi anemia (D'Andrea, 2003). Significantly, cell lines deficient in BRCA2 function exhibit genome instability, hypersensitivity to DNA damaging agents, and a pronounced deficiency in HR (Jasin, 2002). Cytological results indicate that BRCA2 is important for the assembly of DNA damage-induced Rad51 nuclear foci (Yang et al., 2002; Tarsounas et al., 2003). BRCA2 physically interacts with Rad51 through a series of eight copies of a reiterated motif called the BRC repeat. Furthermore, BRCA2 possesses a single-stranded DNA (ssDNA) binding function (Yang et al., 2002) and tightly associates with a small partner protein called DSS1 (Marston et al., 1999), which is also needed for DSB repair by HR (Kojic et al., 2003; Gudmundsdottir et al., 2004). These features of BRCA2 are consistent with the possibility that it acts as a recombination mediator by promoting the assembly of the Rad51 presynaptic filament. This report details the progress made towards elucidating the molecular function of BRCA2 in HR.

Body

(i) Expression and purification of hBRCA2-derived polypeptides - Human BRCA2 contains eight BRC repeats in its middle portion and a DNA binding domain (DBD) toward the carboxyl terminus as deduced from biochemical and crystallographic studies done with the mouse BRCA2 orthologue (Yang et al., 2002) (Figure 1A). Due to its enormous size (3,418 amino acid residues) neither others nor we have been able to purify full-length BRCA2. However, since several BRCA2-like molecules from organisms such as *Ustilago maydis* and *C. elegans* are of much smaller size and contain only a single BRC, we wished to test the premise that by fusing selected BRC repeats and the DBD, we would be able to synthesize a form of human BRCA2 protein that is capable of physical and functional interactions with human Rad51 (hRad51) protein. Accordingly, we constructed a polypeptide that

consists of the BRC3 and BRC4 repeats and the DBD derived from human BRCA2 (Figure 1B). To facilitate the purification of the BRC3/4-DBD polypeptide, a Thioredoxin/S-protein–tag and hexahistidine–tag were attached to its amine and carboxy-termini, respectively. The BRC3/4-DBD fusion protein expressed in *E. coli* is soluble, and a simple four-step purification procedure encompassing: nickel-NTA beads, hydroxyapatite column chromatography, ammonium sulfate precipitation and S-tag affinity pulldown, was devised to purify it to near homogeneity (Figure 1C,D). The identity of the purified protein was established by immunoblot analysis and MALDI-TOF (Figure 1E). Up to 200 μg of the purified BRC3/4-DBD can be obtained from 400 g of *E. coli* cell paste. We also expressed and purified (1) a GST fusion protein that contains the BRC3 and BRC4 repeats (GST-BRC3/4) and (2) a $(His)_6$-tagged form of the DBD (data not shown).



E

Residues in BRCA2	Peptide sequence	e value
1414-1434	TEQNIKDFETSDTFFQTASGK	$1x10^{-11}$
1446-1472	IVNFFDQKPEELHNFSLNSELHSDIRK	$2x10^{-18}$
1493-1512	ESVPVGTGNQLVTFQGQPER	$2x10^{-10}$
2556-2570	NAESFQFHTEDYFGK	$8x10^{-7}$
2869-2888	IQEEFEEHEENTTKPYLPSR	$7x10^{-12}$
3105-3132	FWIDLNEDIIKPHMLIAASNLQWRPESK	$1x10^{-21}$

Figure 1 Purification of BRC3/4-DBD. **(A)** Schematic outlining the functional domains in BRCA2. The DNA binding domain (DBD) consists of a helical domain (HD) and three oligo-nucleotide binding (OB1, OB2, and OB3) folds that confer the ability to interact with DSS1 and ssDNA. **(B)** The human BRCA2-derived polypeptide BRC3/4-DBD harbors two of the BRC repeats (BRC3 and BRC4 within residues 1409-1596) linked to the DBD (residues 2477 to 3194). BRC3/4-DBD contains thioredoxin (Trx) and S tags at its amino-terminus and a (His)6 tag at its carboxyl-terminus. **(C)** Schematic of the chromatographic procedure devised for BRC3/4-DBD purification. **(D)** Purified BRC3/4-DBD was analyzed by SDS-PAGE and Coomassie Blue staining (2 μg in lane 2) or immunoblotting with anti-histidine antibodies (200 ng in *lane 3*). **(E)** Results from MALDI-TOF analysis of purified BRC3/4-DBD. Six representative fragments and their corresponding e values are shown.

(ii) Interaction of BRC3/4 and BRC3/4-DBD with hRad51 - Taking advantage of the affinity tags on BRC3/4-DBD, GST-BRC3/4, and DBD, we used several *in vitro* pulldown assays to investigate whether BRC3/4-DBD binds hRad51. First, purified hRad51 was incubated with BRC3/4-DBD, and the resulting protein complexes were captured on anti-S antibody agarose beads through the S-tag on BRC3/4-DBD. As shown in Figure 2A, BRC3/4-DBD bound hRad51. Affi-gel beads containing crosslinked hRad51 were used to verify the role of the BRC repeats in hRad51 binding. As anticipated, BRC3/4-DBD and GST-BRC3/4 bound the hRad51 beads with considerable avidity, while DBD did not (Figure 2B). We also examined whether the *E. coli* RecA protein and human Dmc1 (hDmc1) protein interact with the two BRCA2-derived polypeptides. No association of either RecA or hDmc1 with either BRCA2-derived polypeptide was seen (Figure 2A,C and data not shown). We conclude that BRC3/4-DBD associates with hRad51 in a specific manner, through the BRC repeats.

(iii) BRC3/4-DBD binds ssDNA preferentially - The mouse Brca2 protein possesses three OB (oligonucleotide-oligosaccharides binding) folds that endow it with DNA binding ability.

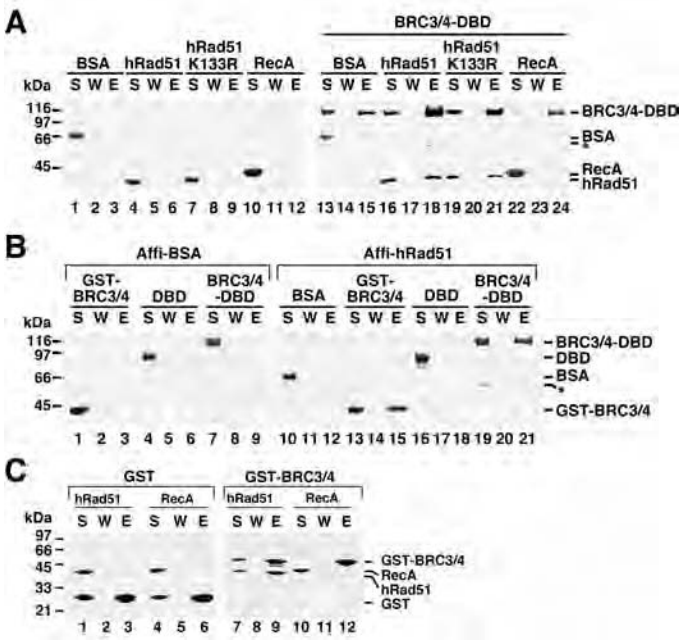


Figure 2 BRC3/4-DBD binds hRad51. **(A)** BSA, hRad51, hRad51K133R, and RecA were mixed with anti-S protein agarose beads in the absence (*lanes 1-12*) or presence of BRC3/4-DBD (*lanes 13-24*) and then subject to affinity pulldown with anti-S agarose beads. **(B)** GST-BRC3/4, DBD, and BRC3/4-DBD were subject to affinity pulldown with Affi-beads conjugated to BSA (Affi-BSA; *lanes 1-9*) or hRad51 (Affi-Rad51; *lanes 10-21*). **(C)** Pulldown assays using GST or GST-BRC3/4 on glutathione Sepharose confirmed that BRC3/4 binds Rad51 but not RecA. The supernatant (S), wash (W), and SDS eluate (E) from the above reactions were analyzed by SDS-PAGE and Coomassie Blue staining. A proteolytic product of BRC3/4-DBD is marked by the asterisk.

We wished to verify that the BRC3/4-DBD species that we constructed from the human BRCA2 sequence also has DNA binding capability. To do this, increasing amounts of the purified BRC3/4-DBD was incubated with either ^{32}P-labeled 80mer ssDNA (Figure 3A) or ^{32}P-labeled 80bp duplex (Figure 3B) obtained by hybridizing the former oligonucleotide to its complement. The reaction mixtures were resolved in non-denaturing polyacrylamide gels, which were dried and then analyzed in the phosphorimager to reveal shifting of the DNA species by BRC3/4-DBD. Consistent with published data obtained with the mouse Brca2 DBD, BRC3/4-DBD binds DNA and with a distinct preference for the ssDNA species (Yang et al., 2002). As expected, when we incubated BRC3/4-DBD with the mixture of ssDNA and dsDNA, it first shifted all of the ssDNA substrate before binding the dsDNA (Figure 3C,D). The DBD alone also bound DNA with a similar preference for the ssDNA species and we could verify GST-BRC3/4 without the DBD does not possess any DNA binding activity (data not shown). Taken together, the data clearly show that the human BRCA2 protein harbors a DNA binding function that is highly specific for ssDNA.

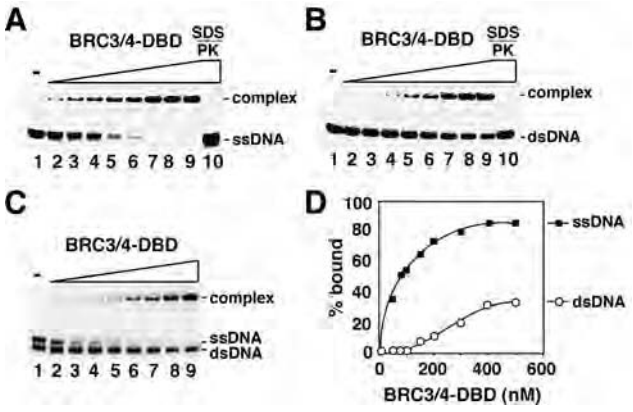


Figure 3 BRC3/4-DBD has high affinity for ssDNA. **(A)** & **(B)** Increasing amounts of purified BRC3/4-DBD (20-500 nM, *lanes 2-9*) was incubated with 30 nM of ^{32}P-labeled ssDNA or dsDNA and then analyzed. Treatment of the nucleoprotein with SDS and proteinase K (SDS/PK) released the DNA substrate (*lane 10*). **(C)** BRC3/4-DBD (50 to 500 nM) was incubated with the mixture of ^{32}P-labeled ssDNA and dsDNA and then analyzed. **(D)** The results from the experiment in C are plotted.

(iv) BRC3/4-DBD targets Rad51 to ssDNA - In mediating HR, Rad51 protein must first assemble onto ssDNA to form a helical nucleoprotein filament, which provides the catalytic center for the pairing of recombining DNA molecules (Sung et al., 2003). The assembly of the Rad51-ssDNA nucleoprotein filament is hampered by (1) slow nucleation of Rad51 onto ssDNA, (2) competition for binding site on the DNA by RPA, and (3) sequestration of Rad51 on dsDNA. Given that BRC3/4-DBD physically interacts with hRad51 and binds ssDNA with avidity, we tested to see if BRC3/4-DBD could target hRad51 to ssDNA. To address this question, we incubated hRad51 with a mixture of dsDNA and biotinylated ssDNA with or without BRC3/4-DBD, followed by capture of the biotinylated ssDNA on magnetic beads that contains streptavidin, to which the biotin moiety on the ssDNA binds with high affinity. The magnetic beads and the supernatant fractions were treated with SDS and then subject to SDS-PAGE to determine their content of Rad51 and BRC3/4-DBD (Figure 4A). The results showed that inclusion of BRC3/4-DBD increased the percentage of Rad51 associated with the magnetic bead-bound ssDNA (Figure 4B, *lanes 4-5*). Importantly, neither GST-BRC3/4 nor the DBD alone was effective in enhancing the association of Rad51 with ssDNA (Figure 4B, *lanes 6-7*).

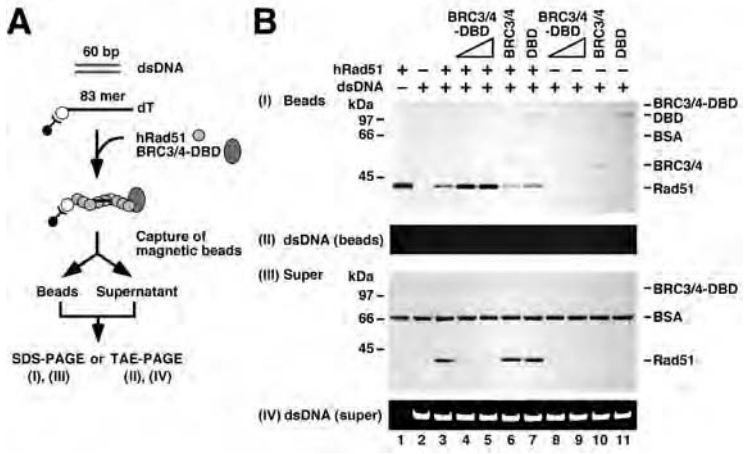


Figure 4 BRC3/4-DBD targets hRad51 to ssDNA. **(A)** Schematic of the assay. Magnetic bead-bound oligo dT was incubated with hRad51, BSA and BRCA2-derived polypeptides, without or with an excess of dsDNA, as indicated. Proteins bound to the oligo dT were captured with a magnet and then eluted with SDS. **(B)** The supernatants (super) and SDS eluates (beads) were analyzed for their protein and DNA contents. While, as expected, the majority of hRad51 was trapped on the dsDNA (*lane 3*), BRC3/4-DBD efficiently targeted hRad51 to the ssDNA (*lanes 4 and 5*). GST-BRC3/4 (BRC3/4) or DBD was ineffective in this regard (*lanes 6 and 7*, respectively).

(v) Recombination mediator activity of BRC3/4-DBD - As a result of competition for binding sites, hRPA can exclude Rad51 from ssDNA and cause a marked suppression of Rad51 recombinase activity (Symington, 2002; Sung et al., 2003). Given that BRC3/4-DBD binds both Rad51 and ssDNA and is capable of targeting Rad51 to ssDNA, we used an oligonucleotide-based assay to ask whether BRC3/4-DBD could act as a recombination mediator by helping overcome the suppressive effect of hRPA. For this assay, a concentration of hRPA sufficient to ablate homologous DNA pairing catalyzed by hRad51 was used. As shown in Figure 5A-B, restoration of homologous DNA pairing was seen upon addition of an amount of BRC3/4-DBD similar to the concentration of the ssDNA template. Full restoration of homologous DNA pairing was seen at a concentration of BRC3/4-DBD that was still ten times lower than that of hRad51, indicative of a catalytic mode of action in BRC3/4-DBD. Control experiments showed that BRC3/4-DBD alone is devoid of DNA

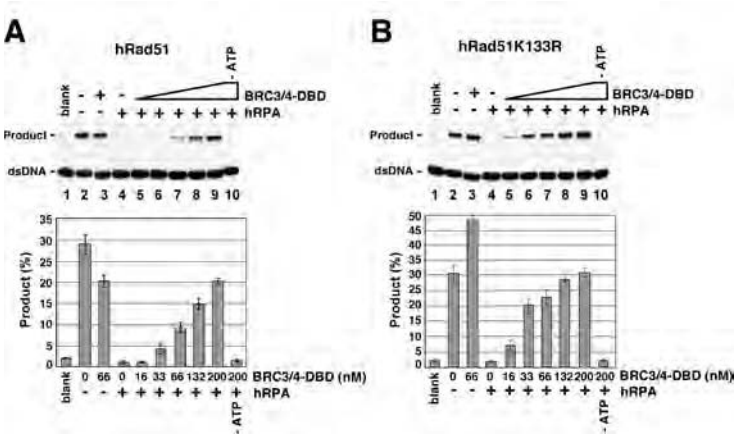


Figure 5 Recombination mediator activity of BRC3/4-DBD. **(A)** & **(B)** Homologous DNA pairing reactions containing hRad51 or hRad51 K133R and varying amounts of BRC3/4-DBD were carried out with ssDNA or hRPA-coated ssDNA, as indicated. The averaged values of results from three independent experiments are presented in the histograms. The no protein control (*lane 1*) is marked as "blank", and ATP was omitted from the reaction in *lane 10*.

pairing activity and that the restored DNA pairing reaction is strictly ATP-dependent (Figure 5). Significantly, neither DBD nor GST-BRC3/4, nor the combination of both of these polypeptides, in the same concentration range of BRC3/4-DBD used, was able to overcome the suppressive effect of hRPA (Figure 6A-C), indicating that both domains are required in *cis* for recombination mediator activity.

(vi) Examination of BRC3/4-DBD recombination mediator function by electron microscopy - We employed electron microscopy (EM) to further examine the recombination mediator activity of BRC3/4-DBD. In the EM-analysis, hRad51-ssDNA filaments exhibited the characteristic striations noted in previous studies (Figure 7A), while the nucleoprotein complexes of RPA with ssDNA had a non-descript appearance (Figure 7B). In congruence with the biochemical data, when the 150-mer ssDNA was first coated with hRPA before hRad51 was added, we saw an abundance of hRPA-ssDNA complexes but very few presynaptic filaments (Figure 7C). The inclusion of BRC3/4-DBD at a concentration sufficient to restore homologous pairing with hRPA-coated ssDNA template to near the uninhibited level, led to robust presynaptic filament formation (Figure 7C). Thus, the results of the EM analyses are also clearly indicative of a recombination mediator function of BRC3/4-DBD.

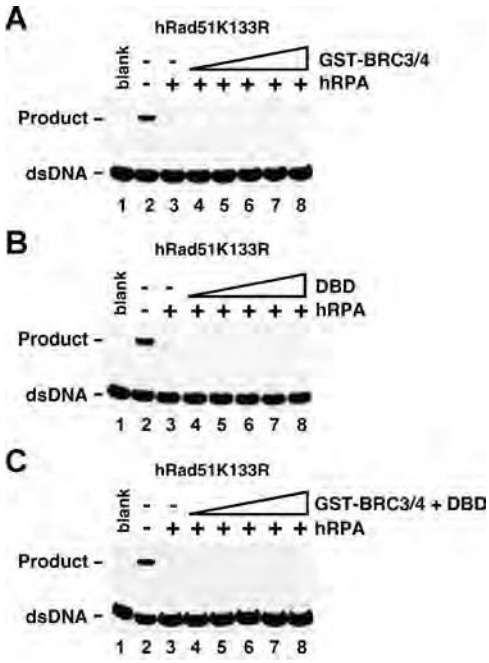


Figure 6. Experiments showing that neither GST-BRC3/4 **(A)** nor DBD **(B)** or a mixture of the two polypeptides **(C)** is capable of overcoming the inhibitory effect of hRPA on homologous DNA pairing by hRad51 K133R. The concentrations of the BRCA2 polypeptides were 25, 50, 100, 150, and 200 nM.

(vii) Specificity and versatility of the recombination mediator activity - As shown earlier, BRC3/4-DBD has no affinity for *E. coli* RecA protein (Figure 2A). We tested whether RecA-mediated homologous DNA pairing is responsive to BRC3/4-DBD. As with hRad51, hRPA suppresses DNA pairing catalyzed by RecA. Importantly, little or no restoration of RecA-mediated homologous DNA pairing was seen

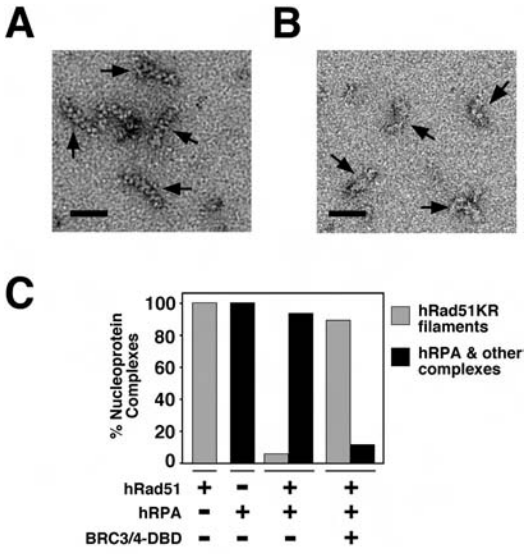


Figure 7. Examination of recombination mediator activity by electron microscopy. **(A)** Examples of hRad51 K133R-ssDNA nucleoprotein filaments and **(B)** examples of hRPA-ssDNA complexes formed on the 150-mer ssDNA substrate are shown. Control experiments confirmed the requirement for ssDNA in the formation of the hRad51 K133R filaments and hRPA-containing complexes. The bar in black denotes a length of 50 nm. **(C)** Data quantification of reaction mixtures that contained either hRad51 K133R and hRPA or hRad51 K133R, hRPA, and BRC3/4-DBD. Over 1,500 nucleoprotein complexes were counted to determine the relative abundance of the hRad51 K133R-ssDNA filaments.

upon addition of BRC3/4-DBD (Figure 8A). We also examined whether BRC3/4-DBD could overcome the inhibitory effect of *E. coli* single-strand binding protein, SSB, on hRad51-mediated homologous DNA pairing. As shown in Figure 8B, addition of BRC3/4-DBD was able to completely reverse the inhibitory effect of SSB on the DNA pairing reaction.

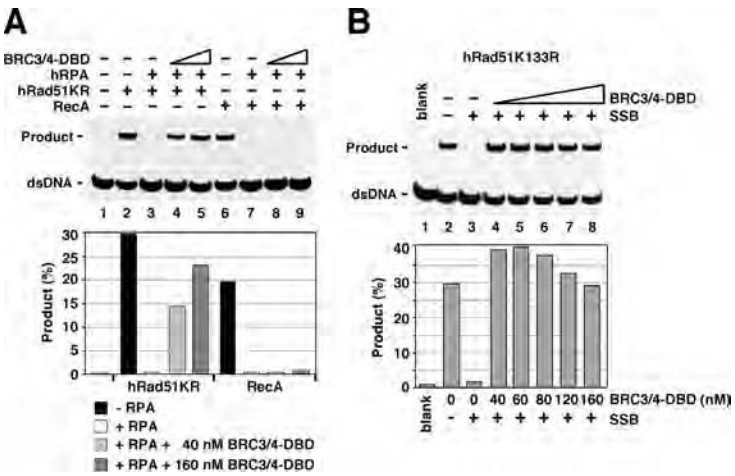


Figure 8. Specificity and versatility of the BRC3/4-DBD recombination mediator activity. **(A)** BRC3/4-DBD is unable to overcome the suppressive effect of hRPA on RecA-mediated homologous DNA pairing (*lanes 6 to 9*). hRad51 K133R (hRad51KR) was included in this analysis as control (*lanes 2 to 5*). **(B)** *E. coli* SSB attenuates homologous DNA pairing by hRad51 K133R, and this suppressive effect is overcome by BRC3/4-DBD (*lanes 4-9*). The no protein control (*lane 1*) is marked as "blank". The results are presented in the histograms.

(viii) Assembly of the BRC3/4-DBD/DSS1 complex - The stability and functionality of BRCA2 is dependant on the small (70 amino acid residues), highly acidic protein DSS1 (Kojic et al., 2003; Gudmundsdottir et al., 2004). It has been reported that DSS1 binds BRCA2 in the vicinity of the DBD domain, through a series of hydrophobic and ionic contacts. The high acidity of DSS1 could mimic the phosphodiester backbone of DNA and, as such, may function to attenuate the affinity of RPA, thereby promoting the exchange of RPA by the BRCA2-Rad51 complex (Figure 9). To begin

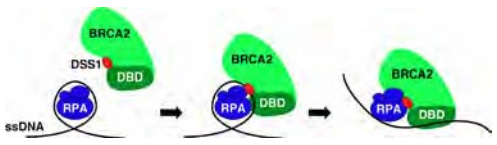


Figure 9. Model depicting DSS1 helping mediate RPA eviction from ssDNA by acting as a DNA mimic .

testing this hypothesis, DSS1 was expressed in *E. coli* as a GST fusion protein and we devised a procedure to purify it to near homogeneity (Figure 10A). The identity of the purified protein was established by immunoblot analysis and MALDI-TOF (data not shown). In addition, we were able to assemble and purify a stoichiometric complex of GST-DSS1 with BRC3/4-DBD (which harbors the DSS1 binding site)(Figure 10B).

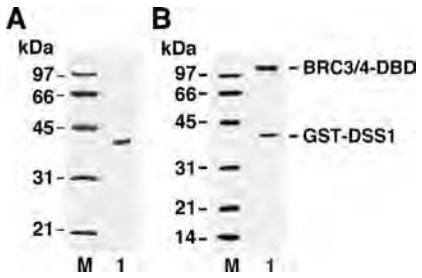


Figure 10. Purification of GST-DSS1. **(A)** Purified GST-DSS1, and **(B)** GST-DSS1 co-purified with BRC3/4-DBD were analyzed by SDS-PAGE.

(ix) DSS1 binds and attenuates the DNA binding activity of RPA - Our preliminary studies indicate that purified GST-DSS1 binds hRPA and attenuates DNA binding by hRPA (Figure 11A & C), consistent with the hypothesis put forth. Complex formation and the attenuation of DNA binding are specific for hRPA, as they are not seen with *E. coli* SSB (Figure 11B & D).

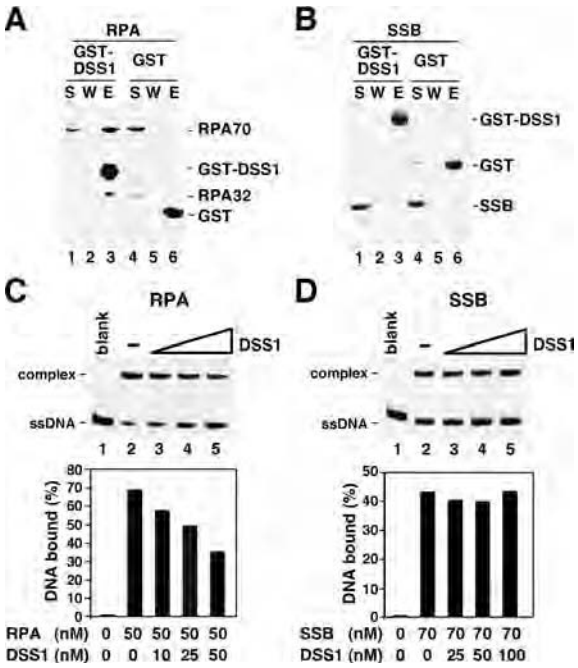


Figure 11. Interactions of GST-DSS1 with RPA. RPA **(A)** or *E. coli* SSB **(B)** was incubated with GST-DSS1 or GST and then mixed with glutathione Sepharose to capture a possible protein complex. The supernatant (S), wash (W), and SDS eluate (E) from the reactions were subjected to SDS-PAGE analysis. RPA **(C)** or SSB **(D)** was pre-incubated with the indicated concentrations of GST-DSS1 before the incorporation of the ssDNA substrate (^{32}P-labeled oligo dT-30). The reaction mixtures were run in polyacrylamide gels, which were dried and subjected to phosphorimaging analysis to visualize the DNA substrate and nucleoprotein species. The data were quantified and presented in the two histograms.

Key Research Accomplishments

• Expressed and purified a polypeptide that harbors the BRCA2 DNA binding domain and two of the BRC repeats, BRC3 and BRC4

• Demonstrated that the BRCA2-derived polypeptide, BRC3/4-DBD interacts with Rad51 through the BRC motifs, and binds DNA with a distinct preference for ssDNA

• Demonstrated by both biochemical means and electron microscopy that BRC3/4-DBD is capable of nucleating Rad51 on ssDNA, and can act as a recombination mediator by enabling Rad51 to utilize RPA-coated ssDNA as a recombination substrate.

• Showed specificity for BRC3/4-DBD for nucleating hRad51 onto ssDNA occupied by ssDNA-binding proteins.

• Expressed and purified DSS1 and a stoichiometric complex of BRC3/4-DBD/DSS1

Reportable Outcomes

Krejci, L., Van Komen, S., Li, Y., Villemain, J., Reddy, M. S., Klein, H., Ellenberger, T., Sung, P. (2003) DNA helicase Srs2 disrupts the Rad51 presynaptic filament. Nature 423:305-9.

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Conclusions

A role for BRCA2 in HR and DNA repair is well established, however, relatively little is known about the protein's function at a mechanistic level. The studies summarized in this report help to clarify the role of BRCA2 as a recombination mediator, specifically functioning to promote the assembly of the Rad51 presynaptic filament. This experimental system should be valuable for systematically dissecting the HR functions of BRCA2 and its associated proteins such as DSS1. Comprehending the manner in which BRCA2 modulates Rad51 activity and the functional integrity of the HR machinery could very well pave the way for devising new strategies in breast cancer diagnosis, prevention, and treatment.

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